19

Cell Wall Fractionation for Yeast and Fungal Proteomics

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Summary

The cell wall is an external envelope shared by yeasts and filamentous fungi that defines the interface between the microorganism and its environment. It is an extremely complex structure consisting of an elastic framework of microfibrillar polysaccharides (glucans and chitin) that surrounds the plasma membrane and to which a wide array of different proteins, often heavily glycosylated, are anchored in various ways. Intriguingly, these cell wall proteins (CWPs) play a key role in morphogenesis, adhesion, pathogenicity, antigenicity, and as a promising target for antifungal drug design. However, the CWPs are difficult to analyze because of their low abundance, low solubility, hydrophobic nature, extensive glycosylation, covalent attachment to the wall polysaccharide skeleton, and high heterogeneity. We describe a typical procedure of cell wall fractionation to isolate and solubilize different CWP species from yeasts and filamentous fungi according to the type of linkages that they establish with other wall components and under suitable conditions for following reproducible proteomic analyses. CWPs retained noncovalently or by disulfide bonds are first extracted from isolated yeast or fungal cell walls by detergents and reducing agents. Subsequently, CWPs covalently linked to or closely entrapped within the internal glucan-chitin network are sequentially released either by mild alkali conditions or by enzymatic treatments first with glucanases and then with chitinases. This strategy is a powerful tool not only for obtaining an overview of the sophisticated cell wall proteome of yeasts and filamentous fungi, but also for characterizing mechanisms of incorporation, assembly and retention of CWPs into this intricate cellular compartment and their interactions with structural wall polysaccharides.

Key Words: Cell wall; cell wall proteins; fractionation; fungus; GPI proteins; PIR proteins; proteomics; yeast.

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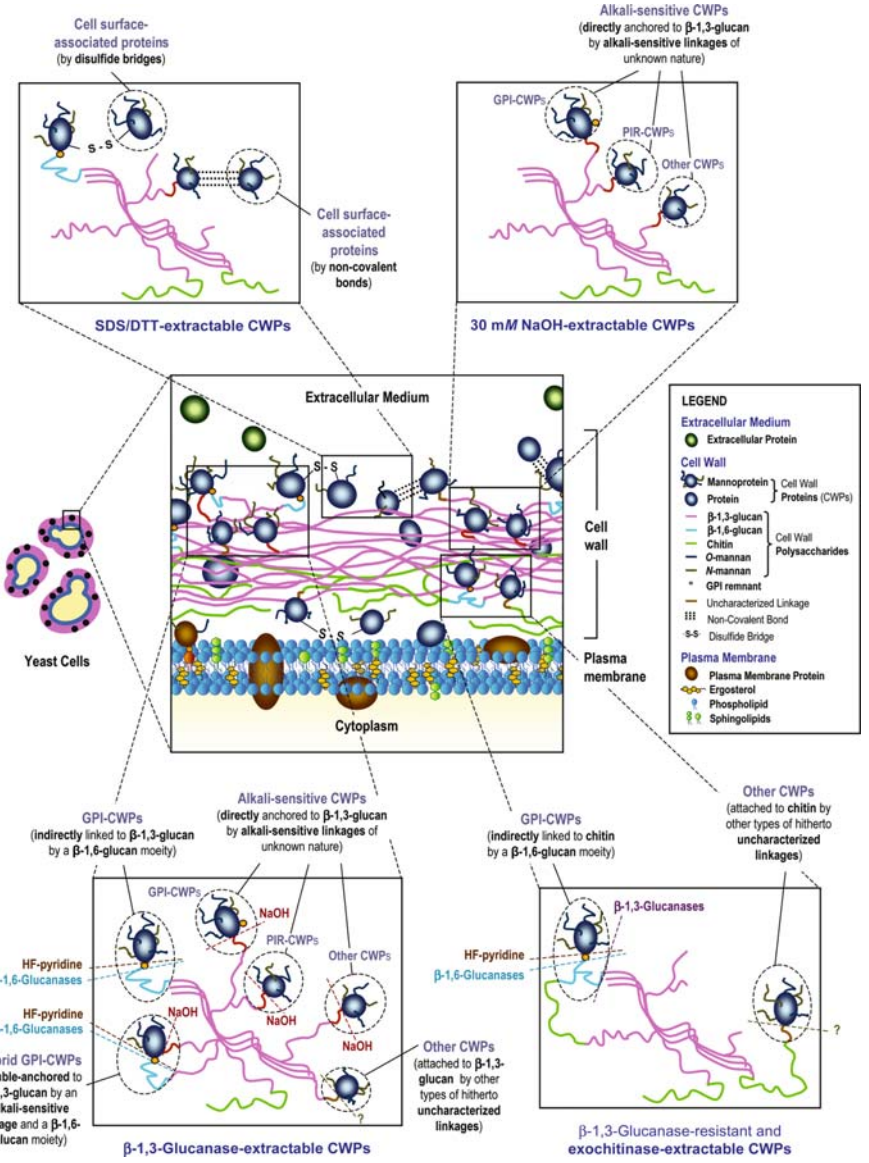

Fig. 1. Schematic representations of the cell wall from *S. cerevisiae* and *C. albicans*, and molecular modules of CWPs solubilized by the present procedure of cell wall fractionation. The cell wall of *S. cerevisiae* and *C. albicans* basically consists of β-1,3 and β-1,6-glucans, chitin, mannoproteins and proteins. β-1,3-glucan and chitin form an elastic microfibrillar polysaccharide skeleton surrounding the plasma membrane to which mannoproteins are attached through β-1,6-glucan, alkali-sensitive bonds, and/or other hitherto uncharacterized linkages. Cell wall proteins (CWPs; mannoproteins and

1. Introduction

The cell wall is an intricate structure common to yeasts and filamentous fungi that surrounds the plasma membrane and is strategically placed at the interface between the cell and its environment, including the host ***(1–3)***. This external envelope, accountable for 20–30% of the cell dry weight, is essential for the survival of the microorganism. In fact, it is involved in many vital functions, such as physical protection, osmotic stability, selective permeability barrier, immobilized enzyme support, cell-cell interactions (e.g., cell recognition and adhesion) and morphogenesis, to name but a few. In pathogen fungi, this cellular compartment further takes an active part in virulence, pathogenicity, antigenicity, immunomodulation of the immune response, and adhesion to host substrates ***(2)***. Most importantly, its essential nature and its fungal specificity (given its absence in mammalian cells) interestingly make the cell wall an attractive target site (*see* **Note 1**) to design antifungal drugs with selective toxicity for human pathogen fungi, such as *Candida albicans* or *Aspergillus fumigatus*, among others ***(4–6)***.

Although the cell wall structure and organization have been investigated most extensively in the prototype yeast *Saccharomyces cerevisiae*, a similar molecular model is also applicable for other ascomycetes, and in particular for *C. albicans*, a dimorphic fungus capable of growing either in yeast form or as hyphae (*see* **Note 2**) ***(1,7,8)***. The cell wall of *S. cerevisiae* is mainly composed of glucans (with β-1,3 and β-1,6 linkages), chitin (*N*-acetylglucosamine polymers), and proteins (often highly *O*- and/or *N*-mannosylated) interconnected by covalent and/or non-covalent bonds, leading to an elevated complexity (*see* **Fig. 1**). β-1,3-glucan, the major component of the cell-wall (electron-transparent) inner layer, forms an elastic three-dimensional microfibrillar framework, encircling the cell, to which other wall constituents are covalently anchored. Chitin is often cross-linked to the β-1,3-glucan microfibrillar backbone on its inner side (close to the plasma membrane) and, to a lesser extent, to short side-chains of β-1,6-glucan. This *N*-acetylglucosamine polymer presents low levels (*see* **Note 2**), except in the budding neck ring, in the primary septum, in and around the bud scars, or under stress conditions. Both β-1,3-glucan and chitin (structural wall polysaccharides) provide mechanical strength and elasticity

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Fig. 1. proteins) can also be loosely associated, either by non-covalent bonds or through disulfide bridges, with other covalently linked CWPs. *See* **Introduction** for further information. The *callouts* depict details for potential mechanisms of CWP retention into the cell wall on the basis of the procedure of cell wall fractionation described in this chapter to isolate and solubilize different CWP species from yeasts and filamentous fungi.

to the cell wall. β-1,6-glucan, a flexible minor wall component, interconnects certain cell wall proteins (CWPs), the so-called glycosyl phosphatidylinositol (GPI)-CWPs, with β-1,3-glucan (~90% of GPI-CWPs) or chitin (~10% of GPI-CWPs) through a phosphodiester bridge in their GPI remnant (*see* **Note 3**). The CWPs are mostly located on the outside of this β-1,3-glucan-chitin network (i.e., at the cell-wall electron-dense outer layer) and, in minor amounts, throughout the cell wall, determining its porosity. These CWPs can be:

1. Loosely associated, either noncovalently or through disulfide bonds, with other cell wall components. This group of CWPs comprises (1) soluble precursor forms of covalently linked CWPs, (2) proteins related to the biosynthesis and modulation of wall constituents, such as β-1,3-glucosyltransferase (Bgl2p), β-exoglucanase (Exg1p) and chitinase (Cts1p), and (3) noncanonical proteins "classically" considered to be confined to the intracellular compartment because they lack the conventional secretory signal sequence ***(2,9–11)***. Nevertheless, the mechanism by which these nonconventional proteins are targeted to the cell surface remains enigmatic ***(11–13)***. This array of loosely associated proteins is commonly found at the cell surface but also, in a smaller ratio, in the cell-wall inner layers. These CWPs can be extracted using detergents and reducing agents (*see* **Fig. 1**).
2. Covalently linked to β-1,3-glucan:
   a. Directly *via* an alkali-labile linkage (speculatively through a *O*-linked side-chain), such as PIR-CWPs (CWPs with internal repeats). The PIR-CWPs are highly *O*-mannosylated proteins with one or more internal repeat regions, a *N*-terminal signal peptide, a Kex2 proteolytic processing site, and a *C*-terminal sequence with four cysteine residues at highly conserved positions ***(1,14–17)***. They are normally located in the cell-wall inner layer ***(18)***. Other CWPs belong to this category are also present in the yeast cell walls (*see* the following and **Fig. 2**). This type of CWPs can be solubilized under mild alkali conditions or by enzymatic treatment with β-1,3-glucanases but not with β-1,6-glucanases (*see* **Figs. 1–3**).
   b. Indirectly by a β-1,6-glucan moiety through their GPI remnant, such as GPI-CWPs. The GPI-CWPs are highly *O*-glycosylated proteins with an *N*-terminal signal peptide, a *C*-terminal GPI anchor addition signal, and serine- and threonine-rich regions (*see* **Note 3**) ***(1,7,17,19)***. These CWPs are predominantly placed in the cell-wall outer layer. This group of CWPs can be released either by enzymatic treatment with β-1,3 or β-1,6-glucanases or by using hydrofluoric acid (HF)-pyridine, which cleavages the phosphodiester bond in the GPI remnant ***(20)*** (GPI-CWP 1 in **Figs. 1–3**).
3. Covalently anchored to chitin by a β-1,6-glucan moiety *via* their GPI remnant, such as some GPI-CWPs ***(21,22)***. This type of CWP-polysaccharide complex is largely found in the lateral walls or under stress conditions. These CWPs can be

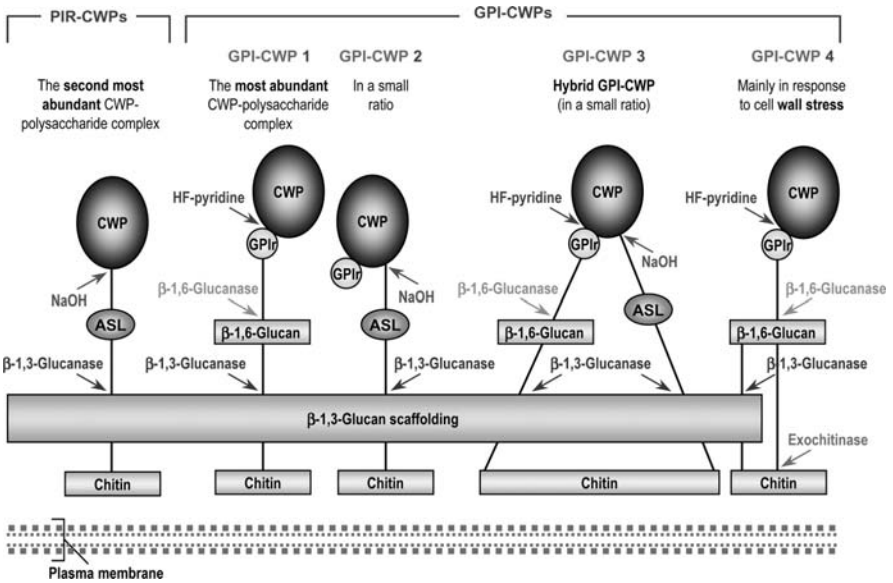

Fig. 2. Schematic representations of the different known types of covalently linked CWPs in *S. cerevisiae* and *C. albicans*, and the most commonly used methods to solubilize them from isolated cell walls. These diagrams are based on information from references ***(1,14,18-21,23)***. *See* **Introduction** for further details. PIR-CWPs, a small number of hybrid GPI-CWPs (*e.g.*, Cwp1p ***(23)***; GPI-CWPs 2 and 3) at acidic pHs, and other CWPs ***(25,32)*** are directly attached to β-1,3-glucan through an alkali-sensitive linkage (ASL). GPIr denotes GPI remnant, and ASL alkali-sensitive linkage.

extracted either by enzymatic treatment with chitinases or β-1,6-glucanases or by using HF-pyridine (GPI-CWP 4 in **Figs. 1–3**).

However, it is unsurprising that other types of linkages, hitherto uncharacterized, among CWPs and structural wall components are also present in the yeast cell wall (*see* **Fig. 1**). Be that as it may, there is no doubt that the molecular model of the yeast cell wall is even more sophisticated than that outlined above. This is because certain CWPs can simultaneously be retained into the β-1,3-glucan/chitin skeleton in various ways. For instance, the Cwp1p, a *S. cerevisiae* GPI-CWP, is double-anchored to the β-1,3-glucan framework both through an alkali-sensitive linkage and by its β-1,6-glucan moiety, playing an important role in stress response ***(23)***. Hence, two additional GPI-CWP-polysaccharide complexes are defined (GPI-CWPs 2 and 3 in **Figs. 2** and **3**).

Taking into account the special architecture and nature of the cell wall, the isolation and solubilization of CWPs from this complex cellular compartment is not therefore an evident and easy affair. Indeed, CWPs from yeasts

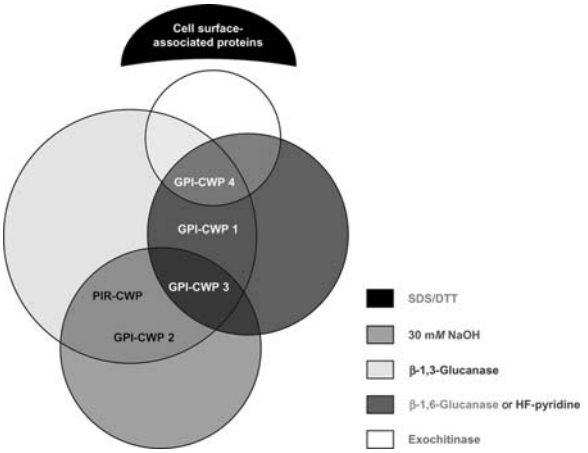

Fig. 3. Venn diagram summarizing the most commonly used methods to extract the different known types of CWPs in *S. cerevisiae* and *C. albicans*. This chart is based on data from references ***(1,10,14,18–21,23)***. The GPI-CWP numbers refer to those indicated in **Fig. 2**.

and filamentous fungi are tricky enough to resolve by two-dimensional electrophoresis (2-DE) gels, because of their low abundance, low solubility, hydrophobicity, high heterogeneity, extensive glycosylation (especially, *O*- and/or *N*-mannosylation), and covalent attachment to the wall polysaccharide (β-1,3-glucan/chitin) skeleton ***(11,24)***. These problems can, at least to some degree, be solved by sequential solubilization of CWPs on the basis of the type of attachments that they establish to other cell wall components ***(11,17,25)***. This procedure implies breakage of the covalent linkages between CWPs and wall polysaccharides (*see* **Chapter 20**). Intriguingly, cell wall fractionation is an appropriate paradigm system to:

1. Reduce the intricacy of the cell wall.
2. Enrich samples for CWPs and thus increase the detection of low-abundance species by removing the most abundant soluble gene products.
3. Enhance the solubility of large, low abundance, and/or hydrophobic CWPs.
4. Define (map and identify) the proteins that make up the cell wall (the cell wall proteome), and elaborate a comprehensive and integrated view of the complex CWP composition. The CWP resolution can be increased using the cell wall fractionation procedure described here, because the heterogeneous population of protein species present in the yeast and fungal cell envelope can be distributed over several 2-DE gels ***(11)***.
5. Characterize mechanisms of incorporation, assembly and retention of CWPs into the cell wall.
6. Elucidate the CWP interactions with cell wall polysaccharides.

7. Study protein-protein interactions or regulatory networks exclusive to the cell wall.
8. Monitor abnormal protein expression localized to this external envelope.
9. Discover novel diagnostic/prognostic markers, antifungal targets and/or therapeutic candidates for human mycoses.

This chapter will integrate a typical procedure of cell wall fractionation to extract different CWP species from isolated cell walls of yeasts or filamentous fungi according to their interactions with other wall components. The resulting selectively enriched CWP fractions can then directly be (1) analyzed by 2-DE (*see* **Note 4**) and mass spectrometry (MS) ***(11,17)*** or (2) digested with trypsin, followed by liquid chromatography (LC) in tandem with MS analyses ***(25)*** to circumvent some of the difficulties associated with *in*-gel digestion of the heavily glycosylated CWPs ***(12)***. The purity and quality of these enriched fractions of CWPs should be screened by using *bona fide* markers both of the cell wall and of intracellular compartments before carrying out any interpretation of the results. However, the unambiguous evidence for their cell wall location will only be established after (1) their *in situ* immunolocalization (i.e., immunoelectron microscopy or immunofluorescence studies) and/or (2) the use of tagged fusion proteins (e.g., c-*myc*-tag or green fluorescence protein (GFP) fusion proteins) based on the fusion of the protein in question to modified versions of extracellular enzymes that rely on a detectable phenotype.

2. Materials

Growth media, solutions and buffers should be sterilized by autoclaving before use when working under sterile conditions. Their labile components should be sterilized separately using a 0.22-µm filter (Millipore, Bedford, MA) and then added to the other autoclaved ingredients. All solutions and buffers should be prepared with ultrapure water, as provided by Nanopure or Milli-Q 18 MΩ/cm resistivity systems (Millipore), and precooled when the procedure is carried out at 4°C.

2.1. Cell Wall Isolation from Yeasts and Filamentous Fungi

1. Yeast-Peptone-D-glucose (YPD) plates: 1% (w/v) yeast extract (Difco Laboratories, Detroit, MI), 2% (w/v) peptone (Difco), 2% (w/v) D-glucose, 2% (w/v) agar (Difco).
2. YPD medium: 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) D-glucose.
3. Bead mill homogenizer (model MSK; Braun Biotech International GmbH, Melsungen, Germany).
4. Liquid carbon dioxide (CO_2).

5. 0.40- to 0.60-mm chilled, acid-washed glass beads (Sartorius, Goettingen, Germany) (*see* **Note 5**).
6. PMSF stock solution: 0.1 *M* in isopropanol. Dissolve 174 mg of phenylmethylsulfonyl fluoride (PMSF; Fluka, Chelmsford, MA) in a final volume of 10 mL isopropanol, and store at –20°C (*see* **Note 6**). PMSF should be handled with caution because it is **highly toxic**. Weigh this hazardous compound in a fume hood, and wear gloves, goggles and a mask.
7. Lysis buffer: 10 m*M* Tris-HCl, pH 7.4, 1 m*M* PMSF.
8. YPD-chloramphenicol plates: 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) D-glucose, 2% (w/v) agar (Difco), 10-µg/mL chloramphenicol. When the 1-L autoclaved YPD and agar solution cools to ~65°C, add 1 mL of 10× chloramphenicol solution.
9. Chloramphenicol solution (10X): Dissolve 10 mg of chloramphenicol in a final volume of 1 mL ethanol, and sterilize using a 0.2-µm filter.
10. Wash solution A: 1 m*M* PMSF.
11. Wash solution B: 5% (w/v) NaCl, 1 m*M* PMSF.
12. Wash solution C: 2% (w/v) NaCl, 1 m*M* PMSF.
13. Wash solution D: 1% (w/v) NaCl, 1 m*M* PMSF.

2.2. Protein Solubilization from Isolated Yeast and Fungal Cell Walls

2.2.1. By Detergents and Reducing Agents

1. Wash buffer: 50 m*M* Tris-HCl, pH 8.0, 1 m*M* PMSF.
2. Extraction buffer: 50 m*M* Tris-HCl, pH 8.0, 0.1 *M* EDTA, 2% (w/v) SDS, 10 m*M* DTT (dithiothreitol; *see* **Note 7**).

2.2.2. Under Mild Alkali Conditions

1. Wash solution: 1 m*M* PMSF.
2. Wash buffer: 0.1 *M* sodium acetate, pH 5.5, 1 m*M* PMSF.
3. Extraction solution: 30 m*M* NaOH, 1 m*M* PMSF.
4. Stop solution: acetic acid.

2.2.3. By β-1,3-Glucanase Treatment

1. Wash solution: 1 m*M* PMSF.
2. Wash buffer: 50 m*M* Tris-HCl, pH 7.5, 1 m*M* PMSF.
3. Extraction buffer: 1,500 U Quantazyme *ylg*™ (Quantum Biotechnologies Inc, Montreal, Canada; *see* **Note 8**) per gram of wet weight of cell walls, in 2 mL of a solution containing 50 m*M* Tris-HCl, pH 7.5, 10 m*M* DTT, 1 m*M* PMSF (*see* **Note 9**).
4. Stop solution: 10% SDS.

2.2.4. By Exochitinase Treatment

1. Wash solution: 1 m*M* PMSF.
2. Wash buffer: 50 m*M* sodium phosphate buffer, pH 6.3.
3. Extraction buffer: 0.3 U exochitinase (Sigma, St. Louis, MO) per gram of wet weight of cell walls, in 2 mL of a solution containing 50 m*M* sodium phosphate buffer, pH 6.3 (*see* **Note 10**).
4. Stop solution: 10% SDS.

2.3. Protein Precipitation.

1. 100% Trichloroacetic acid (TCA) solution: Dissolve 100 g of TCA in sufficient water to yield a final volume of 100 mL (*see* **Note 11**). TCA should be handled with caution, because it is **extremely caustic**. Protect eyes and avoid contact with skin when working with TCA solutions.
2. Acetone precooled to –20°C.
3. Neutralizing solution: 0.1 *N* NaOH.

3. Methods

The protocols described below outline a typical procedure of cell wall fractionation to isolate and solubilize different CWP species from yeasts and filamentous fungi on the basis of the type of linkages that they establish with other wall components. This method involves (1) cell homogenization by physical disruption techniques, (2) isolation of cell walls by differential centrifugation, (3) sequential solubilization of CWPs from isolated cell walls using different chemical agents (detergents, reducing agents, and alkalis) and enzymes (glucanases and chitinases), and (4) precipitation of the resulting selectively enriched CWP fractions under suitable conditions for subsequent proteomic analyses. A flowchart of the strategy presented here is shown in **Fig. 4**. This is based on earlier methods described by Kapteyn et al. ***(20,21)*** and Mrsa et al. ***(26)***, and on recent modifications reported by Pitarch et al. ***(11)*** that have proved effective at properly extracting CWPs from *S. cerevisiae* and *C. albicans*. Nevertheless, adaptation of growth and extraction conditions may be required for other species of yeasts and filamentous fungi.

3.1. Cell Wall Isolation from Yeasts and Filamentous Fungi (see Note 12)

Although cell disintegration can be accomplished using a wide variety of techniques, mechanical breakage of cells using glass beads is certainly one of the most quick and reliable procedures to disrupt the cell walls and plasma membranes of yeasts and filamentous fungi (*see* **Note 13**). After cell disruption,

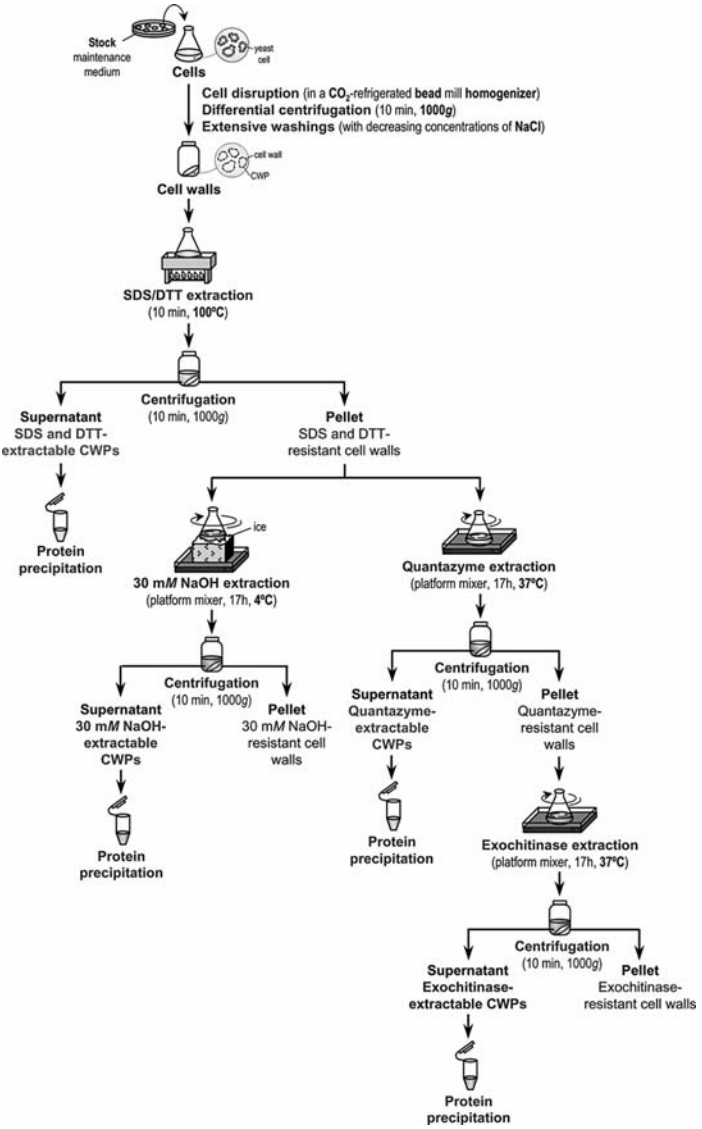

Fig. 4. Flowchart of a typical procedure of cell wall fractionation to isolate and solubilize different CWP species from yeasts and filamentous fungi according to the type of attachments that they establish to other cell wall components.

cell walls can be separated from other cytosolic and membranous components by differential centrifugation of the cell homogenate at relatively low speed (*see* **Note 14**).

1. Grow yeast or fungal cells on an YPD plate (stock maintenance medium) at 30°C for 2 days. Use a single colony to inoculate 50 mL of YPD (or selective) medium in a 250-mL flask (*see* **Note 15**), and grow overnight at 30ºC with vigorous rotary shaking (200 rpm).
2. Use this 50-mL preculture to inoculate four 2-L flasks containing 500 mL fresh YPD medium each, and grow at 30°C in a shaking incubator (200 rpm) until the culture reaches early-log phase growth (OD_{600nm} = 0.5–1; *see* **Note 16**).
3. Harvest the cells by centrifugation at 4,500*g* for 5 min (*see* **Note 17**) and discard the supernatant.
4. Resuspend the cell pellet in 200 mL ice-cold water, and centrifuge 5 min at 4,500*g*. Decant the supernatant.
5. Resuspend the cell pellet in 200 mL ice-cold lysis buffer, and centrifuge 5 min at 4,500*g*. Discard the supernatant.
6. Resuspend the cells in 3 volumes of ice-cold lysis buffer, and add 3–4 volumes of 0.5-mm acid-washed glass beads (*see* **Note 18**). Transfer the cell suspension to an appropriate sized shaking flask (*see* **Note 19**).
7. Grind the suspension at maximum speed for 30–60 s using a bead mill homogenizer and cooling with liquid CO_2 (for example, in a CO_2-refrigerated MSK homogenizer), and then place the shaking flask on ice for 1–2 min (*see* **Note 20**). Repeat this step until complete cell breakage. Monitor the degree of cell breakage with a phase-contrast microscope and by plating on YPD-chloramphenicol plates (*see* **Note 21**).
8. Enable the glass beads to settle out and collect the supernatant carefully (*see* **Note 22**). Wash the glass beads with ice-cold lysis buffer and collect the washings until they are clear (*see* **Note 23**). Pool the supernatant and all the washings.
9. Centrifuge the pooled supernatant and washings (cell homogenate) at 1,000*g* for 10 min at 4°C. Discard the supernatant carefully (*see* **Note 24**).
10. Resuspend the isolated cell walls in 200 mL ice-cold wash solution A. Centrifuge 10 min at 1,000*g* and at 4°C. Carefully decant the supernatant. Repeat this step two more times.
11. Resuspend the cell walls in 200 mL ice-cold wash solution B. Centrifuge 10 min at 1,000*g* and at 4°C. Carefully decant the supernatant. Repeat this step four more times (*see* **Note 25**).
12. Resuspend the walls in 200 mL ice-cold wash solution C. Centrifuge 10 min at 1,000*g* and at 4°C. Carefully remove the supernatant. Repeat this step four more times.
13. Resuspend the pellet in 200 mL ice-cold wash solution D. Centrifuge 10 min at 1,000*g* and at 4°C. Carefully decant the supernatant. Repeat this step four more times (*see* **Note 26**).
14. Resuspend the walls in 200 mL ice-cold wash solution A. Centrifuge 10 min at 1,000*g* and at 4°C. Carefully discard the supernatant. Repeat this step two more times using preweighed centrifuge bottles.
15. Weigh the wet wall pellet (*see* **Note 27**).

3.2. Protein Solubilization from Isolated Yeast and Fungal Cell Walls

CWPs can then be solubilized sequentially from isolated cell walls by a wide variety of reagents (*e.g.*, detergents, reducing agents, alkalis, and hydrolytic enzymes, among others) in connection with their attachments to other wall components (*see* **Fig. 4**). All procedures are performed at 4°C with prechilled solutions, reagents and apparatus (*see* **Note 28**) unless otherwise indicated.

3.2.1. By Detergents and Reducing Agents

Detergents, such as sodium dodecyl sulfate (SDS) or *n*-octylglucoside, can be used to extract CWPs that are associated noncovalently with other wall components. The use of reducing agents, such as dithiothreitol (DTT) or β-mercaptoethanol (βME), (1) enables the solubilization of CWPs that are loosely associated, either by disulfide bridges or through non-covalent bonds, with other covalently linked CWPs, (2) results in an increase in the cell wall porosity, and (3) facilitates the subsequent action of wall degrading enzymes (*see* **Figs. 1** and **4**).

1. Resuspend the purified cell walls (prepared as described in Subheading 3.1) in 200 mL ice-cold wash buffer. Centrifuge 10 min at 1,000*g* and carefully decant the supernatant (*see* **Note 24**).
2. Add 5 mL of extraction buffer for each wet gram of cell walls and resuspend. Boil the cell wall suspension at 100°C for 10 min, and centrifuge 10 min at 1,000*g*.
3. Collect the supernatant and store the pellet.
4. Precipitate the supernatant (containing SDS/DTT-extractable CWPs) and store at –80°C (*see* **Note 29** and **Fig. 4**).
5. Repeat **step 2** with the stored pellet and carefully discard the supernatant (*see* **Note 30**).
6. Weigh the wet wall pellet (containing SDS/DTT-resistant cell walls) and divide it equally between two tubes (*see* **Fig. 4** and **Notes 27** and **31**). Store them for further extractions (*see* **Subheadings 3.2.2**, **3.2.3** and **3.2.4**).

3.2.2. Under Mild Alkali Conditions

Treatment of SDS/DTT-resistant cell walls under mild alkali conditions (using 30 m*M* NaOH) results in the extraction of CWPs linked to β-1,3-glucan through an alkali-sensitive linkage (of unknown nature) by the β-elimination process (1, 14–17). PIR-CWPs, some GPI-CWPs, and other CWPs belong this group (*see* **Figs. 1–4**).

1. Resuspend one tube of the purified SDS/DTT-resistant cell walls (prepared as described in **Subheading 3.2.1**) in 200 mL ice-cold wash solution. Centrifuge 10 min at 1,000*g* and carefully decant the supernatant (*see* **Note 24**). Repeat this step two more times.
2. Resuspend the walls in 200 mL ice-cold wash buffer. Centrifuge 10 min at 1,000*g* and carefully discard the supernatant. Repeat this step five to seven more times.

3. Add 4 mL of ice-cold extraction solution for each wet gram of cell walls and resuspend. Incubate the cell wall suspension at 4°C for 17 h with gentle shaking (*see* **Note 32**).
4. Stop the chemical reaction by adding neutralizing amounts of acetic acid (*see* **Note 33**).
5. Centrifuge 10 min at 1,000*g* and collect the supernatant (containing alkali-sensitive CWPs). Precipitate or dialyze the clear supernatant (*see* **Note 33**) and store at –80°C.

3.2.3. By β-1,3-Glucanase Treatment

β-1,3-glucanases (commercially available) can be used to extract CWPs covalently anchored to β-1,3-glucan. This group of CWPs released from SDS/DTT-resistant cell walls by these wall hydrolytic enzymes contains (1) GPI-CWPs, which are indirectly attached to β-1,3-glucan, via β-1,6-glucan, through a phosphodiester bridge and can alternatively be solubilized either by β-1,6-glucanases (*see* **Note 34**) or by using HF-pyridine, (2) alkali-sensitive CWPs (including PIR-CWPs and some GPI-CWPs, among others), which are anchored to β-1,3-glucan through uncharacterized linkages (perhaps by a *O*-linked side-chain) and can also be released under mild alkali conditions (using 30 m*M* NaOH; *see* **Subheading 3.2.2**), and (3) other CWPs linked to β-1,3-glucan through other types of hitherto unidentified bridges (*see* **Figs. 1–4**).

1. Resuspend the other tube of the purified SDS/DTT-resistant cell walls (prepared as described in **Subheading 3.2.1**) in 200 mL ice-cold wash solution. Centrifuge 10 min at 1,000*g* and carefully decant the supernatant (*see* **Note 24**). Repeat this step two more times.
2. Resuspend the walls in 200 mL ice-cold wash buffer. Centrifuge 10 min at 1,000*g* and carefully remove the supernatant. Repeat this step five to seven more times.
3. Add 2 mL of extraction buffer for each wet gram of cell walls and resuspend (*see* **Note 34**). Incubate the cell wall suspension at 37°C for 17 h with gentle shaking.
4. Stop the enzymatic reaction by adding the stop solution at a final concentration of 0.4% (w/v) and heating at 100°C for 3–5 min.
5. Centrifuge 10 min at 1,000*g*.
6. Collect the supernatant (containing β-1,3-glucanase-extractable CWPs) and store the pellet (containing SDS/DTT and β-1,3-glucanase-resistant cell walls) for further extractions (*see* **Subheading 3.2.4**).
7. Precipitate the clear supernatant and store at –80°C.

3.2.4. By Exochitinase Treatment

Enzymatic treatment of the SDS/DTT- and β-1,3-glucanase-resistant cell walls with exochitinases leads to the solubilization of CWPs covalently anchored to chitin. These comprise (1) a small subgroup of GPI-CWPs, which

are indirectly linked to chitin, via β-1,6-glucan, through their GPI remnant and can also be extracted either by β-1,6-glucanases or by using HF-pyridine, and (2) other CWPs attached to chitin through other types of hitherto uncharacterized linkages (*see* **Figs. 1–4**).

1. Resuspend the SDS/DTT and β-1,3-glucanase-resistant cell walls (prepared as described in **Subheading 3.2.3**) in 200 mL ice-cold wash solution. Centrifuge 10 min at 1,000*g* and carefully decant the supernatant (*see* **Note 24**). Repeat this step two more times.
2. Resuspend the walls in 200 mL ice-cold wash buffer. Centrifuge 10 min at 1,000*g* and carefully discard the supernatant. Repeat this step five to seven more times.
3. Add 2 mL of extraction buffer for each wet gram of cell walls and resuspend. Incubate the cell wall suspension at 37°C for 17 h with gentle shaking.
4. Stop the enzymatic reaction by adding the stop solution at a final concentration of 0.4% (w/v) and heating for 3–5 min at 100°C.
5. Centrifuge 10 min at 1,000*g* and collect the supernatant (containing β-1,3-glucanase-resistant and exochitinase extractable CWPs).
6. Precipitate the clear supernatant and store at –80°C.

3.3. Protein Precipitation.

The different selectively enriched CWP fractions obtained in the **Subheading 3.2** should be concentrated and desalted before carrying out further proteomic analyses. These can be (1) dialyzed against a volatile buffer and dried, or (2) precipitated with TCA as described below (*see* **Note 35**), among other methods.

1. Add 1/9th the total volume of the protein sample of an ice-cold 100% TCA solution to a final concentration of 10%.
2. Mix thoroughly and incubate on ice for 30 min.
3. Centrifuge the suspension at 10,000*g* for 15 min, and discard the supernatant (*see* **Note 36**).
4. Wash protein pellet twice with cold acetone to remove residual TCA.
5. Air dry for 30 min.
6. Add neutralizing amounts of 0.1*N* NaOH (*see* **Note 37**) and store at –80°C.

4. Notes

1. Supporting this enterprise, β-1,3-glucan is targeted by a new antifungal drug class in recent clinical use, i.e., echinocandins (including caspofungin and micafungin), which blocks the biosynthesis of this cell wall polysaccharide (4–6).
2. It must be borne in mind that unlike *S. cerevisiae*, many filamentous fungi contain further α-glucans and a high chitin content in their cell walls ***(1)***.
3. The GPI-proteins are translocated into the endoplasmic reticulum (ER), where (1) the *N*-terminal signal peptide (secretion signal necessary to enter the classical

ER-Golgi secretory pathway) is cleaved, (2) the *C*-terminal GPI anchor addition signal is replaced with a GPI anchor, and (3) *O*- and/or *N*-linked core glycosylation takes place. Remarkably, further glycosylation also then occurs in the Golgi apparatus. These GPI-anchored proteins are directed through the secretory pathway to the outer side of the plasma membrane, where they are attached through their *C*-terminal GPI anchors (*see* **Fig. 5**). Intriguingly, some of them are released from the plasma membrane by cleavage of their GPI anchors, resulting in GPI anchor remnants (a truncated, lipidless GPI anchors) ***(1,19,27,28)***. These proteins are then covalently incorporated into the cell wall, by the attachment of their GPI remnants to β-1,6-glucan ***(19)***.

4. It is convenient to use gradient gels that enable protein separation up to at least 500 kDa, because the extensive glycosylation (especially, *O*- and/or *N*-mannosylation) of a large proportion of CWPs results in extremely high apparent molecular masses on SDS-PAGE or 2-DE gels ***(7)***.
5. The size of the glass beads is crucial to achieve an efficient cell disruption. Optimal bead size for spores is 0.1 mm and for yeast and mycelia 0.5 mm.
6. The PMSF can also be solubilized in ethanol, methanol and 1,2-propanediol. It is unstable in aqueous solution. PMSF is added to reduce possible proteolytic processes. It inhibits serine proteases (e.g., trypsin, chymotrypsin, and thrombin) and thiolproteases (e.g., papain).
7. 40 m*M* β-mercaptoethanol may be substituted for 10 m*M* DTT. β-mercaptoethanol or DTT should be added just before use.
8. Quantazyme *ylg*™ is a recombinant yeast β-1,3-glucanase purified from *E. coli* that is completely free of protease, endo- and exonuclease activity. It is highly stable in solution for months at 4°C, maintaining its whole activity.
9. The use of reducing agents facilitates the ability of Quantazyme *ylg*™ to degrade the cell wall β-1,3-glucan. 40 m*M* β-mercaptoethanol or 10 m*M* cysteine may be substituted for 10 m*M* DTT.
10. Exochitinase is a cell wall lytic enzyme isolated from *Serratia marcescens* that catalyzes the progressive degradation of chitin, starting at its nonreducing end. This preparation contains phosphate buffer salts and shows an optimum pH of 6.0.
11. Because TCA is very hygroscopic, the whole content of a newly opened TCA bottle should be used to prepare the TCA stock solution.
12. Perform all procedures from this subheading until isolated cell walls are obtained under sterile conditions. Use sterile centrifuge bottles.
13. Mechanical cell breakage using a bead mill homogenizer is considered the technique of choice for disrupting cells with cell walls, especially spores, yeasts and fungi, but it also works successfully with algae, bacteria, plant, and animal tissue culture in suspension. Cell disruption takes place by the crushing action of the glass beads, which are vigorously agitated by shaking or stirring, after crashing with the cells. The Braun MSK cell homogenizer combines two types of motions in the shaking flask: rotation and tumbling. For small-scale preparations, a Fast-Prep cell breaker (Q-Biogene, Carlsbad, CA) can be used in lieu

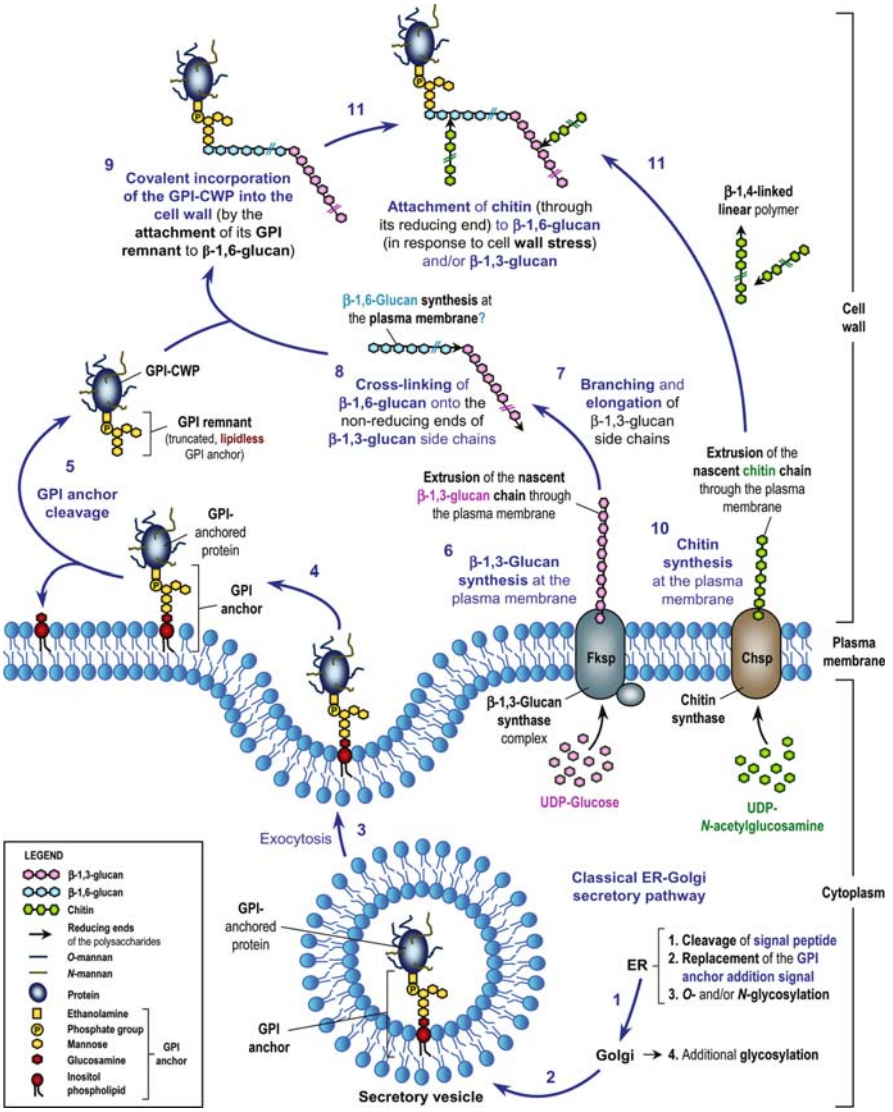

Fig. 5. Putative model of the incorporation of GPI-anchored proteins into the cell wall. This is based on information from references ***(19,27,28)***. Their GPI anchor is processed at the plasma membrane leading to a GPI remnant (a truncated, lipidless GPI anchor). This is cross-linked to β-1,6-glucan when these GPI proteins are covalently incorporated into the cell wall. *See* **Note 3** for further details.

of the Braun MSK cell homogenizer. Cell breakage of filamentous fungi can alternatively be achieved by (1) grinding freeze-dried mycelium in a mortar and pestle, (2) grinding mycelium in liquid nitrogen, or (3) homogenizing thick cell pastes. When handling liquid nitrogen, use insulated gloves, avoid any potential contact because of the risk of frostbite, and never utilize glass containers because they may break.

14. Differential centrifugation in sucrose density gradients may alternatively be used to isolate the cell wall fraction.
15. Liquid cultures should be grown in a flask that is at least 4–5 times larger than the culture volume.
16. It is important that the yeast culture is in early-log phase growth ($\sim OD_{600nm} = 0.5–1$) because it is easier to disrupt their cell walls with the bead mill homogenizer than those close to or in stationary phase growth. It must be borne in mind that the composition of the cell wall changes with the growth stage and culture conditions (growth temperature, external pH, oxygen levels, and composition of the growth medium ***(1,29)***), among others. Hence, it may be necessary to adjust the cell density according to the specific objectives of the experiment.
17. Overall, yeasts are successfully harvested by centrifugation, whereas vacuum-assisted filtration, rather than centrifugation, is often preferred for harvesting filamentous fungi.
18. The wet weight (in grams) of the cell pellet is nearly equal to the packed cell volume (in milliliters). Add about 3 mL of ice-cold lysis buffer for each wet gram of cell pellet and resuspend.
19. The shaking flasks can be made of glass or stainless steel. The latter are better at transferring heat. It is essential to exclude all air from the shaking flask before the cell breakage to avoid foaming and denaturation of proteins.
20. It is extremely important that the temperature of the cell suspension remains at 4°C during the cell disruption to prevent heat inactivation and denaturation of proteins. Use liquid CO_2 to cool the protein sample during cell breakage.
21. This procedure is carried out until complete cell breakage, which will vary with yeast/fungal strain and growth stage. The degree of cell breakage should be examined:

    a. Before proceeding: by observing cell lysis under a phase-contrast microscope.
    b. After proceeding: by plating an aliquot of the cell suspension after and before cell disruption on YPD-chloramphenicol plates and growing at 30°C. The ratio of CFUs after to before cell breakage is then calculated to estimate the efficiency of cell disruption and, therefore, potential intracellular contamination in the subsequent steps. Failure of cells to grow on YPD-chloramphenicol plates should be evidenced.

22. The supernatant (cell homogenate) and following washings (residual cell homogenate) can be collected (1) by decanting after allowing the glass beads to settle out by gravity, or (2) by straining through a perforated tube or "strainer"

(*see* **Fig. 6**). To perform the last method, transfer the cell homogenate containing the glass beads to a plastic tube and make holes of a diameter less than 0.4–0.6 mm in its bottom with a flamed needle to obtain a "strainer." After straining the cell homogenate off, collect it and wash the glass beads with ice-cold lysis buffer. Collect the washing, and wash again until the collected washings are clear. Pool the cell homogenate and all the washings. The washing step is critical

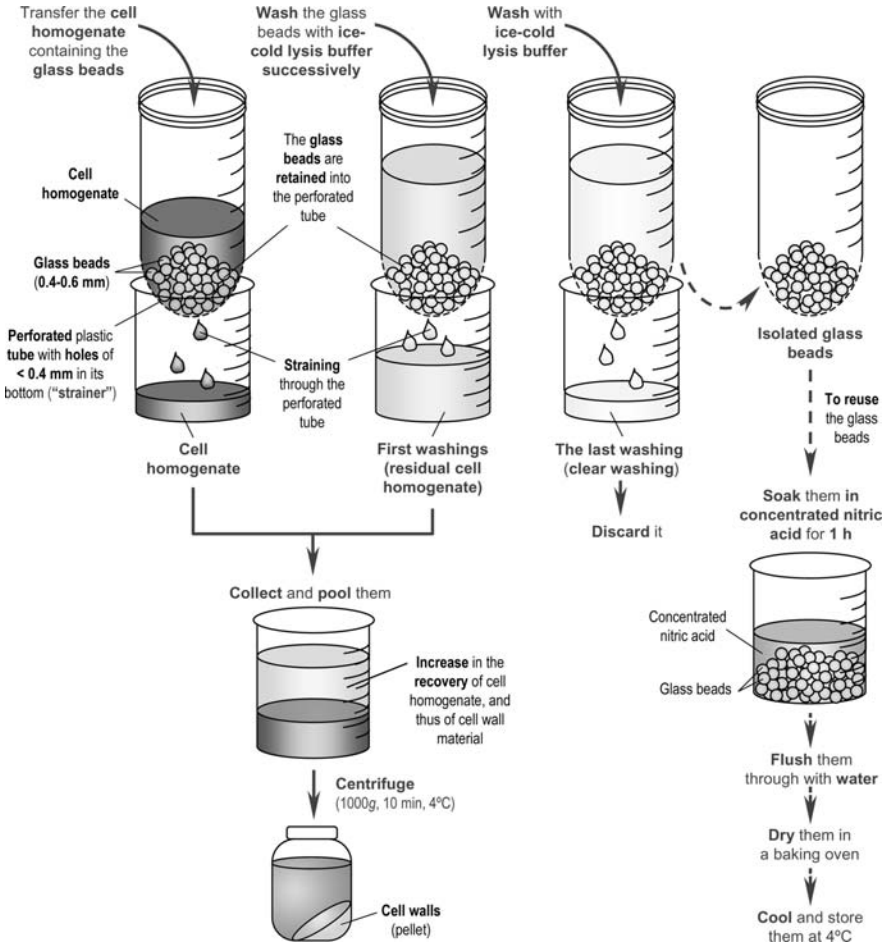

Fig. 6. Proposed procedure for removing the glass beads from the cell homogenate. In the method described here, the cell homogenate is strained through a perforated tube with holes of a diameter less than 0.4–0.6 mm in its bottom, whereas the glass beads (with a diameter of 0.4–0.6 mm) are retained in the perforated tube or "strainer" (*see* **Note 22**). Successive washings with ice-cold lysis buffer should then be performed to increase the recovery of cell homogenate, and thus of cell wall material.

to avoid any loss of material while removing the glass beads from the cell homogenate.

23. To reuse the glass beads, rinse them by soaking in concentrated nitric acid for 1 h, and then flush through with water. Dry them in a baking oven, cool, and store at 4°C (*see* **Fig. 6**).
24. It is important to decant the supernatant carefully, because the cell-wall pellet is less compact than the preceding cell pellets. In general, care should be taken during the following steps of this procedure (especially during decanting of the washings) to prevent any loss of cell walls, and therefore of CWPs. Be that as it may, this potential loss of protein material does not interestingly result in a preferential deficiency of certain CWP species or families.
25. The purpose of extensively washing the isolated cell walls with solutions of decreasing concentrations of NaCl is to remove potential extracellular, membranous and/or cytosolic protein contaminants that can be adhered to them through nonspecific ionic interactions (*see* **Fig. 7**). The number of washing steps will therefore rely on the objectives of the experiment.
26. A further washing step with 0.1 *M* Na_2CO_3 overnight at 4°C under gentle shaking may reduce potential cytoplasmic contamination that is found into microsomes, because this treatment allows them to be opened and washed ***(30,31)***.
27. The wet weight (in grams) of cell walls in the pellet can be calculated by taking the weight of the centrifuge bottle (which has been previously weighed) away from the total weight (i.e., the weight of wall pellet plus centrifuge bottle).
28. Centrifugation must be performed in refrigerated centrifuges at 4°C, with prechilled rotors, to avoid undesirable proteolytic activity.
29. This fraction is not a pure preparation of cell wall, but rather is enriched in CWPs (loosely associated with other wall components) and may potentially contain a small amount of membrane proteins.
30. This extra step is important to remove any remaining SDS/DTT-extractable CWPs and potential membranous components from the isolated cell walls, which will be used in subsequent CWP extraction steps (*see* **Fig. 4**).
31. This pellet, containing SDS/DTT-resistant cell walls, is divided equally between two tubes to independently extract alkali-sensitive CWPs and β-1,3-glucanase-extractable CWPs in the following steps (*see* **Fig. 4**).
32. It is convenient to place the cell wall suspension into a container with ice in a cool room at 4°C (*see* **Fig. 4**).
33. The chemical reaction must be stopped by acid neutralization. This can be performed:

    a. by adding neutralizing amounts of acetic acid to the wall suspension. The clear supernatant containing alkali-sensitive CWPs should then be (*i*) precipitated by adding nine volumes of ice-cold methanol ***(18)*** or (*ii*) dialyzed against water or 20 m*M* bis-Tris, pH 6.0 ***(32)***.
    b. by subsequent protein precipitation of the clear supernatant containing alkali-sensitive CWPs with TCA (*see* **Subheading 3.3**).

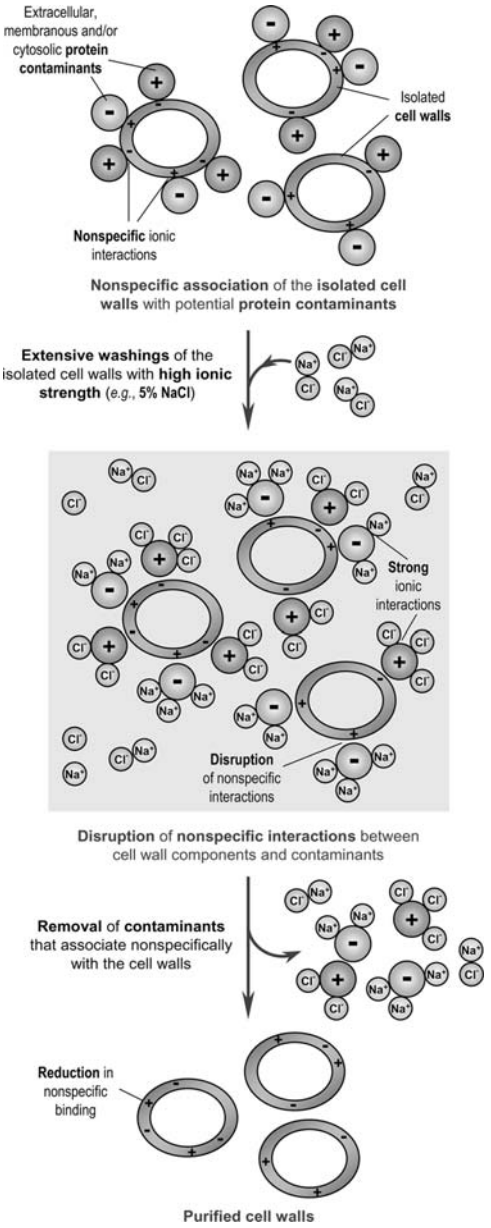

Fig. 7. Basic principle of the procedure used for washing the isolated cell walls of yeasts and filamentous fungi. Potential extracellular, membranous and/or cytosolic protein contaminants that may nonspecifically associate with the isolated cell walls through ionic interactions can be removed under relatively more stringent conditions. Extensive washings of the isolated cell walls with high ionic strength (e.g., 5% NaCl) can disrupt the nonspecific ionic interactions between the isolated cell walls and these putative contaminants.

34. Endo-β-1,6-glucanase isolated from *Trichoderma harzianum* ***(33)*** can be used to release GPI-CWPs (0.8 U/g wet weight of cell walls in 100 m*M* sodium acetate pH 5.5) ***(18,20)***. Subsequently, β-1,6-glucanase-digested cell walls can be treated with Quantazyme or ice-cold 30 m*M* NaOH to extract (1) the β-1,6-glucanase-resistant PIR-CWPs, (2) the β-1,6-glucanase-resistant GPI-CWPs (GPI-CWPs linked to the β-1,3-glucan through a alkali-sensitive linkage), and (3) hybrid GPI-CWPs, such as Cwp1p (*see* **Fig. 2**) ***(23)***.
35. The protein concentration should be higher than 100 μg/mL before precipitating with TCA. If the amount of protein precipitated is less than 1 nmole, the protein sample should be concentrated and desalted by other ways (e.g., by ultrafiltration or forced dialysis), because the pellet is imperceptible.
36. The supernatant should be stored in case the protein did not precipitate.
37. Protein preparation can then be resuspended in a small volume of buffer suitable for the subsequent analytical procedures (e.g. sample buffer for SDS-PAGE or 2-DE).

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